

FLORIDA DEPARTMENT OF AGRICULTURE & CONSUMER SERVICES  
DIVISION OF PLANT INDUSTRY  
BUREAU OF CITRUS BUDWOOD REGISTRATION

**CITRUS BUDWOOD TESTING MANUAL**

The Citrus Nursery Stock Certification Program, Rule Chapter 5B-62, F.A.C., requires testing of all sources of propagating material. The specific requirements are stated in Rule 5B-62.012, F.A.C. Testing procedures, sample collection, and laboratory certification procedures necessary to comply with the Rule 5B-62.012, F.A.C., are in this manual.

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## I. INTRODUCTION

This field-testing manual is based on the Florida Department of Agriculture and Consumer Services' Citrus Nursery Stock Certification Program outlined in Rule Chapter 5B-62, F.A.C. The biological indexing testing procedures are based on those in Roistacher's book, "Graft-transmissible diseases of citrus: Handbook for detection and diagnosis", published in 1991 by the Food and Agriculture Organization of the United Nations in Rome.

The testing for all source trees, including foundation source trees, scion trees, and parent trees is outlined in this manual. Diseases that are established as being present in Florida and are graft-transmitted are tested for once every six years, except those that are vectored which are tested for annually. Our indexing program for detection of these virus and viroid diseases is a comprehensive program based on biological indexing and laboratory testing. Laboratory procedures will be based on our tests of recently published procedures using our collection of known pathogen isolates. Testing procedures will be approved by the citrus budwood technical advisory committee.

## II. SAMPLE COLLECTION AND STORAGE PROCEDURES

- A. Samples shall be collected by an individual authorized by the Division of Plant Industry (DPI).
- B. Fresh tissue samples shall be collected from each of four to six quadrants of each source tree and stored in an insulated cooler for shipment to the testing facility.
  - 1. Samples will be identified by the DPI identification number and double checked with the block, row and tree numbers (where present).
- C. Samples will be collected once a year and scheduled so that all source trees can be sampled within one year's time.

## III. BIOLOGICAL INDEXING

### A. Citrus Viroids

- 1. Samples for testing must be collected as outlined in II. Sample Collection and Storage Procedures on page 2.
- 2. Biological indexing shall be on an Etrog citron (*Citrus medica*, Arizona 861-S-1) source approved by DPI.
- 3. Biological indexing shall be by graft inoculation with two buds of inoculum on each of a minimum of three test plants. At least one viable bud piece shall be present on each plant for at least six weeks after inoculation.
- 4. The control indicator panel shall consist of: plants inoculated with citrus viroids II and III and healthy control plants. One control indicator panel of 12 plants is required for each group of 1 to 100 samples tested.

5. After inoculation, plants shall be cutback to force new growth. Three growth flushes on the indicator plants shall be observed for viroid symptoms. A minimum of one growth flush after positive symptoms are recorded on viroid-inoculated control indicator plants shall be observed. Plants should be observed for leaf epinasty, stunting, tip browning of leaves, petiole and/or midrib cracking and browning and petiole wrinkle.
6. The recommended temperatures for testing are between 32 - 40° C (90 - 104° F) daytime maximum and between 27 - 30° C (81 - 86° F) nighttime minimum.

**B. Citrus psorosis virus and Concave gum**

1. Samples must be collected as outlined in II. Sample Collection and Storage Procedures on page 2.
2. Biological indexing shall be on a sweet orange (*Citrus sinensis*) source approved by DPI.
3. Biological indexing shall be by graft inoculation with two buds of inoculum on each of a minimum of three test plants. At least one viable bud piece shall be present on each plant for at least six weeks after inoculation.
4. The control panel for biological indexing shall consist of 9 plants. Three plants inoculated with a concave gum/blind pocket isolate and 3 plants inoculated with a Psorosis A isolate and 3 uninoculated plants as healthy controls. One control panel of 9 plants is required for each group of 1 to 100 samples tested.
5. After inoculation, plants shall be cutback to force new growth. Three growth flushes on the indicator plants shall be observed for Psorosis symptoms. A minimum of one growth flush after positive symptoms are recorded on inoculated control indicator plants shall be observed. Plants should be observed for vein flecking and/or vein banding, and/or an oak leaf pattern.
6. The suggested time frame for testing is fall through spring. The daytime maximum is 24 - 27°C (75 - 81°F) and nighttime minimum 17 - 21°C (64 - 70°F).
7. Biological indexing in this manner will also detect the presence of concave gum, blind pocket, crinkly leaf and infectious variegation diseases that are reported in Florida as well as impietratura and cristacortis diseases that have not been reported in Florida.

#### **IV. LABORATORY TESTING**

A. Pathogens tested for will consist of endemic citrus graft-transmitted pathogens deemed by the bureau of citrus budwood and its technical advisory committee to be of economic concern to the citrus industry of Florida. This will include, but not necessarily be limited to: Citrus greening, Citrus tristeza virus, Citrus psorosis virus, Citrus tatter leaf virus, (Apple stem grooving virus), Citrus leaf blotch virus and the Citrus viroids: 1 Bent Leaf viroid (CVd I), CVd II, CVd III, CVd IV, and Citrus exocortis viroid (CEVd).

1. The extraction procedure shall be followed as written in Sieburth et al. 2009, on page 142 under Materials and Methods, referenced in Section V. Pertinent Literature.
2. Healthy citrus tissue, no template controls, and positive controls will be included as necessary. See Section VI. Control Panel for a listing of some of the isolates that may be included.
3. The actual real time polymerase chain reaction (PCR) protocol and cycling times that are followed need to be able to indentify a majority of the isolates in the Florida collection for that pathogen.
4. As one yearly sample will provide test material for that year's testing, testing will be concluded within one calendar year of collection.
5. The primers for real time PCR and reverse transcriptase (RT) PCR are those that are able to indentify a majority of the isolates in the Florida collection for that pathogen.

## V. PERTINENT LITERATURE

- Irey, M., P. Sieburth, R. Brlansky, J. DaGraça, J. Graham, T. Gottwald, J. Hartung, M. Hilf, M. Kunta, K. Manjunath, H. Ling, C. Ramdugu, P. Roberts, M. Rogers, R. Shatters, X. Sun, and N. Wang. 2009. Lessons learned from a comparison and evaluation of multiple HLB testing laboratories employing common and different testing methodologies applied to a common set of samples. Proc. Intl. Res. Conf. on Huanglongbing, Orlando, FL, 1–5 Dec. 2008. (In press.)
- Li, W., J.S. Hartung, and L. Levy. 2006. Quantitative real-time PCR for detection and identification of *Candidatus Liberibacter* species associated with citrus Huanglongbing. J. Microbiol. Meth. 66:104–115.
- Li, W., J. S. Hartung, L. Levy. 2007. Evaluation of DNA amplification methods for improved detection of *Candidatus Liberibacter* species associated with citrus Huanglongbing. Plant Dis. 91:51–58.
- Roistacher, C. N. 1991. Graft-transmissible diseases of citrus: Handbook for detection and diagnosis. Food and Agriculture Organization of the United Nations, Rome. 286 pp.
- Sieburth, P. J., K. G. Nolan, Alderman, S. M. and Dexter, R. J. 2009. Increased Efficiency and Sensitivity for Identifying Citrus Greening and *Citrus Tristeza Virus* using Real-time PCR Testing. Proc. Fla. State Hort. Soc. 122: 122:141–146.

## VI. CONTROL PANEL

Control panels may be comprised of the following isolates:

1. Citrus Tristeza Virus Isolates:

Healthy  
FL-7  
FL-86  
FL-216  
FL-217  
FL-277  
FS-577  
FS-627  
T-1  
T-3  
T-4  
T-11  
T-26  
T-28  
T-30  
T-36  
T-55  
T-66  
T-68

2. Citrus Viroid Isolates:

<u>Isolate</u>	<u>Viroid:</u>
Healthy	None
E-9	CEV
E-10	III
E-11	II, III
E-16b	Ila, III, CEV
X-7	Ilc from Florida

3. Citrus Leaf Blotch Virus:

FS693  
FS694  
FS717

4. Citrus Psorosis Virus:

CRSV-4  
CRSV-6B-1  
FS-714  
FS-716  
FS-717

5. Citrus Tatter leaf Virus (Apple Stem Grooving Virus):
  - CTLV-1
  - CTLV-2
  - CTLV-3
  - CTLV-4
  - CTLV-5
  - FL-202
  - FS-595
  
6. Huanglongbing (Citrus Greening):
  - Field isolates